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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

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## PRIORITY DOCUMENT

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**Blatt 2 der Bescheinigung  
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## HIGH OLEIC/HIGH STEARIC SUNFLOWER OILS

(74)

Field of the Invention

The present invention relates to transgenic and non-transgenic sunflower plants that produce oil having high levels of saturated fatty acids and medium to high 5 levels of oleic acid. More specifically the invention relates to a sunflower plant, seed and the extractable oil having increased stearic acid content and increased oleic acid content relative to the normal sunflower oil 10 level of stearic acid and oleic acid. In addition, the invention provides methods for producing the plants, the seeds and the oil.

Background of the Invention

Sunflowers (*Helianthus annuus*) are cultivated 15 for the seeds. Seeds have two principal uses. They are used as confectionery seeds but much more importantly for extraction of oil formed within the sunflower seed. The endogenous oil contains a number of fatty acids. The total fatty acid content for 3 types of sunflowers 20 (normal, high oleic and high palmitic) are listed below. The saturated or unsaturated fatty acid is given as a percentage of weight relative to the total fatty acid content.

25 Table 1

	FATTY ACIDS	CARBON CHAIN	PERCENT WEIGHT
	SATURATED ACIDS		
30	Palmitic	C16:0	5%-7% = (normal) 2%-5% = (high oleic) 22%-40% = (high palmitic)
	Stearic	C18:0	< 10 % but usually 3%-7% (normal, high oleic, high palmitic)
35	UNSATURATED ACIDS		
	Oleic	C18:1	17%-30% = (normal) 9%-12% = (high palmitic) 75%-90% = (high oleic)
40	Linoleic	C18:2	50%-70% = (normal, high palmitic) 2%-10% = (high oleic)

As table 1 indicates, a number of different fatty acid distributions in sunflower oil are known. The table gives the wild type or normal sunflower oil as having a fatty acid distribution of the principle 5 saturated fatty acids ("saturates") as follows: C16:0 levels of 5%-7%, C18:0 levels of <10%, and of the principle unsaturated fatty acids ("unsaturates"): C18:1 levels of 17%-20% and C18:2 levels of 50%-70%. Presently, high oleic and high palmitic are known sunflower oils 10 with changed saturated, unsaturated fatty acid profiles.

The industry has been using both transgenic approaches and mutation approaches to alter the saturated/unsaturated fatty acid profiles in the fatty acid biosynthesis pathway. The percentage of saturated 15 and unsaturated fatty acid present in an altered oil reflect the oil's chemical and physical traits. The chemical and physical traits of the oil are altered to form either more useful oil for the industry and/or a healthier oil for the end user.

20 The last twenty years of sunflower research has produced a healthier oil in high oleic sunflower oil. This research has resulted in the commercial availability of sunflowers having high concentrations of oleic acids (C18:1) as percentage by weight based on the total fatty 25 acid content of the sunflower oil.

Early Russian research into increasing oleic levels in sunflower seeds used mutagenesis to form Pervenets sunflowers having elevated oleic acid levels. Such oleic sunflower material is available to the public. 30 Much, if not all, of the high oleic sunflower germplasm available today is the descendants of the early Russian lines resulting from that research.

Commercially available high oleic sunflower seed includes for example, sunflower variety, Pioneer 35 Hybrid 6661, which is marketed as producing a seed storage oil having a fatty acid composition that includes 85% oleate.

The availability of other high oleic sunflower seed is listed in a number of patent documents. For example, high oleic lines which are believed to be based on the Russian Pervenets sunflowers is described in U.S. 5 Patent No. 4,627,192 and Re-examination certificate B1 4,627,192, issued October 17, 1995, and U.S. Patent No. 4743, 402 and Re-examination certificate B1 No. 4,743,402, issued April 8, 1997 to Fick. These patents list a number of sunflower varieties that are 10 commercially available for breeding purposes that can be licensed under the Fick patents through a company called A.C. Humko. Additionally, U.S. Patent No. 4,627,192 indicates that oleic seeds of Sigco 41A, 41b, 853R, 4117b, 273W, 416R are available from the Lubrizol 15 Corporation 29400 Lakewood Blvd., Wickliffe, Ohio (USA) 44092.

In addition to the research, which resulted in increased oleic acid in sunflower oil, there has also been research on increasing the overall industrial 20 usefulness of sunflower oil, usually addressed by increasing the level of the saturated fatty acids in oil. Increasing the saturated fatty acids produces an oil that is more suitable for use in the production of margarine, shortening, other food products, and cosmetics by 25 decreasing the need for substantial amounts of hydrogenation.

Some of this type of research is outlined in Osorio et al., in Crop Sci. 35: 739-42 (1995). This article describes sunflower seeds developed by 30 mutagenesis and traditional breeding to produce seeds with a high stearate content. This type of research is also outlined in PCT application no. EP95/00369 (WO 95/20313) and teaches a sunflower oil with increased stearic acid content. One way to obtain this oil is to 35 access the deposited seeds. Another way is to repeat the process taught in the patent by treating parent seed with a mutagenic agent to induce one or more mutations in the stearic acid biosynthesis pathway. This process resulted

in an increased production of stearic acid in the sunflower oil in a range between 12% and up to 35% by weight of stearic acid related to the total amount of fatty acid in the oil. High stearic acid producing seeds 5 discussed in this patent are under deposit in the American Type Culture Collection (ATCC). Sunflower seeds identified as "CAS-3" have a average stearic acid content of 25% by weight, related to the total amount of fatty acids in the oil. These seeds were deposited on December 10 14, 1994 with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va 20110-2209, U.S.A. under deposit accession number ATCC 75968. And sunflower seeds identified as "CAS-4" having an average stearic acid content of 15% by weight, related to the total 15 amount of fatty acids in the oil, were deposited on December 14, 1994 with the American type Culture Collection, 10801 University Boulevard, Manassas, Va 20110-2209, U.S.A. under deposit accession number ATCC 75969.

20 PCT/EP95/00369 (WO 95/20313) suggests that oil from high stearic lines could be combined with oil from high oleic lines for certain industrial uses. Unfortunately, although this combination of two oils is useful in many instances, there remains a need for a seed 25 that produces high levels of both stearic and oleic fatty acids in the same oil. Particularly since the levels of linoleic acid produced by these stearic acid lines may tend to produce a less desirable profile of fatty acids, than would be produced by a hybrid producing high 30 stearic, high oleic acid.

Traditional breeding and mutagenesis has not been the only tool used to form seeds producing oil with different fatty acid profiles. Increases in stearic acid in oil bearing plants have also been addressed by the 35 introduction of transgenes into the germplasm, to alter the fatty acid biosynthesis pathway of the vegetable oil. The fatty acid biosynthesis in vegetable oil, but more particularly sunflower oil, includes the biosynthesis of

basically two saturates (palmitate, stearate) and two unsaturates (oleate and linoleate). To give a simplified description of the biosynthesis pathway, it is sufficient to say, that palmitate (C16:0) is by enzymatic action 5 chemically modified to form stearate (C18:0), which by enzymatic action is modified to produce oleate (C18:1), that is further modified to form linoleate (C18:2), some minor amounts of arachic (C20:0) and behenic (C22:0) acids are also formed from stearate. In oilseeds the 10 stearoyl-ACP desaturase is the enzyme which introduces the first double bond on stearoyl-ACP to form oleoyl-ACP. Thus, this is an enzyme that assists in the determination of the unsaturation in the C18 length fatty acids.

In U.S. Patent No. 5,443,974 the inhibition of 15 canola enzyme stearoyl-ACP desaturase was described. The stearate levels were increased but the levels of palmitate were basically unaffected. Inhibition of the plant enzyme stearoyl-ACP desaturase in canola was also reported by Knutzon et al., Proc. Natl. Acad. Sci. USA 20 89:2624-28 (1992). These results showed an increase in the level of stearate produced in the canola seed. The research also showed that inhibition by antisense in seeds of canola and soybean, respectively, showed increased stearate. When a plasmid containing a gene 25 encoding for stearoyl-ACP desaturase was placed in canola, this inhibition resulted in both an increase in stearic acid and unfortunately a reduction in the oleate. However, in the soybean this inhibition of stearate resulted in a less dramatic reduction of the oleate. This 30 slower decrease in oleate however may have been a function of the small initial levels of oleate in the soybean. The fatty acid pathway in most oilseed plants appears to be resistant to maintaining both oleic and stearic at elevated levels.

35 PCT/US97/01419 describes increased levels of both stearic acid and palmitic acid in sunflowers through the inhibition of the plant enzyme stearoyl-ACP

desaturase. Palmitic oil is not, however, viewed as being a very healthy oil.

PCT/US96/09486 discloses that sunflower oil levels of both palmitic and oleic acids could be increased. Sunflower seeds identified as "93PMOL040G" and "93PMOL040F" having increased levels of palmitic 21-23% and oleic 61% were deposited with the American Type Culture Collection (10801 University Boulevard, Manassas, Va 20110-2209) having accession nos. 97159 and 97158, respectively. PCT/US96/09486 teaches a sunflower oil that is liquid at room temperature. But the increased palmitic fatty acid level, is alleged to allow the oil to be used in shortening and in margarine with relatively low level of hydrogenation, which leads to a relatively low level of trans-fatty acids in the resulting product. However, the commercial value may be questioned as palmitic oil is not viewed as being a very healthy oil. Thus margarine made with this oil may be substantially less desirable than margarine made with a healthier combination of fatty acids (i.e. stearic acid).

There thus remains a need for a sunflower oil which is healthy and useful for industrial purposes. There is also a need for a sunflower oil that has a balance of good saturates and good unsaturates. There also remains a need for a sunflower oil that is high in unsaturates but has sufficient saturates to be used for margarines or hardstock without high levels of hydrogenation which lead to no trans-fatty acids in the resulting product. There remains a need for a sunflower plant that can produce seed containing oil which is high in oleic acid and in stearic acid with reduced linoleic levels.

Similar problems as described above for sunflower plants exist for other plants having oil producing seeds.

It is therefore an object of the present invention to provide novel plants, in particular sunflower plants, which form seeds which contain an

endogenous oil wherein the levels of stearic acid (C:18) and oleic acid (C:18:1) are provided in an atypical combination.

5 It is further an object of the present invention to provide plants, in particular sunflower plants, that produce seeds having an oil obtainable therefrom that has an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% and more preferably more than 18 wt%.

10 It is further an object of the present invention to provide seeds and lines, in particular sunflower seed and lines, which when crossed form hybrids having the genetic characteristics to yield high levels of oleic acid and high levels of stearic acid content in 15 the oil as compared to oil obtained from wild type seeds.

It is an object of the present invention to provide F1 hybrid seeds having the genetic characteristics to yield plants which form seeds characterised by high levels of oleic acid and high 20 levels of stearic acid content as compared to oil obtained from wild type seeds.

The invention further relates to plants, in particular sunflower plants, produced from seeds according to the invention that can produce parent lines 25 with high stearic acid and high oleic acid levels in their oil. This applies to the seeds and the progeny therefrom obtained after crossing the plants of the invention with each other or with other germplasm having a desirable characteristic.

30 It is another object of the present invention to produce plants, in particular sunflower plants, that may be used in breeding programs aimed at the production of open pollinated or inbred or hybrids having acceptable agronomic traits that are useful in the field 35 environment.

**Summary of the Invention**

According to the present invention it has now been established that a vegetable oil that is rich in stearic and oleic acids, is formed by altering the 5 standard fatty acid biosynthesis described above. By combining a reduced stearate desaturase activity with a good thioesterase activity on stearoyl-ACP in a high oleic line a vegetable oil rich in stearic and oleic acid is formed according to this invention. The invention 10 accumulates stearoyl-ACP inside the plastid, and the thioesterase over stearoyl-ACP activity exports it out of the plastid. This higher stearic acid content is available for TAG biosynthesis. Later, in the cytoplasm the high oleic character avoids the conversion of oleic 15 acid into linoleic acid.

The invention thus relates to plants which form seeds which contain an endogenous oil wherein the levels of stearic acid (C:18) and oleic acid (C:18:1) are provided in a combination that is different from the 20 combination found in existing plants. More in particular such oil comprises an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of said oil. Preferably, the oleic acid content is from 55 to 75 wt% and the 25 stearic acid content is from 15 to 40 wt%. The plants, seeds, oil, meal and crushed seeds of the invention are for example sunflower plants, seeds, oil, meal and crushed seeds.

The plants and seeds of the invention are 30 obtainable by a method comprising:

- a) providing seeds which contain an oil having a stearic acid content of at least 15 wt%;
- b) providing seeds which contain an oil having an oleic acid content of at least 40 wt% and a high level 35 of thioesterase activity;
- c) crossing plants grown from the seeds provided in step (a) and (b);
- d) harvesting the F1 seed progeny.

Preferably, the method further comprises the steps of:

e) planting the F1 progeny seeds to grow plants;

5 f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seed for the presence of a stearic acid content of at least 15 wt%, an oleic acid content of at least 40 wt% and a high level of

10 thioesterase activity;

h) planting seeds having the desired levels of stearic acid content, oleic acid content and thioesterase activity to grow plants;

i) self-pollinating the plants thus grown to 15 produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic acid content, oleic acid content and thioesterase activity are fixed.

The present invention thus broadly relates to 20 high oleic/high stearic vegetable oils. More specifically the present invention relates to the sunflower seeds, the sunflower plants, sunflower meal or crushed seeds, oil obtainable from the seeds and the extracted endogenous sunflower oil, which has a fatty acid profile high in 25 oleic acid and in stearic acid. Still more specifically this invention has a fatty acid profile that also has preferably less than 25 wt% linoleic and more preferably less than 10 wt% linoleic. Additionally the profile of the invention is often selected to have palmitic levels 30 below 21%.

The broad scope the invention relates to the sunflower seeds, the sunflower plants, sunflower meal or crushed seeds obtainable from the seeds and the extracted sunflower oil, contain or produce or has an oleic acid content of more than 40 wt% and a stearic acid content of 35 more than 12 wt% based on the total fatty acid content of the oil. The more preferred invention especially for certain spread uses has oil and the seeds containing the

oil which contain an oleic acid content that is from 55 to 75 wt% relative to total fatty acid content and a stearic acid content from 15 to 40 wt% relative to total fatty acid content. The scope of the invention includes 5 oil and the seeds containing the oil plants capable of forming such oils which have a total level of saturated fatty acids of at least 20 wt%.

The sunflower seed containing oil is usually selected to have a maximum of 10 wt% of the total 10 saturated fatty acid groups in the 2 position as indicated in Table 6. In some instances the invention can have up to 5 wt% of the saturated fatty acid groups in the 2 position. The present invention also includes a sunflower plant capable of producing the sunflower seeds 15 containing such oils.

The present invention also covers the method of obtaining a sunflower oil having an oleic acid content of more than 40 wt% and a stearic acid content of more than 20 12 wt% based on the total fatty acid content of the oil by extracting oil from the seeds. The method preferably includes an extraction process which does not involve a substantial modification of the sunflower oil.

Additionally, in the process of extraction of the oil 25 from the seed there is preferably no substantial chemical or physical modification nor enzymatic rearrangement taking place and preferably no substantial hardening of the oil.

The present invention also includes food products comprising oil obtainable from sunflower seeds 30 having an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of the oil. Food products that are particularly useful for this type of oil include spreads, margarines, shortenings, sauces, ice-cream, 35 soups, bakery products, confectionery products, and the like. In these food products the level of sunflower oil is preferably from 3 to 100 wt% relative to the total oil weight in the product. When used to form a spread

according to the present invention the sunflower oil is preferably used as a hardstock at levels of 5 to 20 wt%.

The present invention also encompasses cosmetic products comprising an oil, in particular a sunflower oil, the oil having an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of the oil. These cosmetic products can preferably contain levels of sunflower oil from 3 to 100 wt%. Some examples of these cosmetic products would include creams, lotions, 10 lipsticks, soap bars and skin or hair oils.

The present invention also includes a process for selecting Helianthus annuus plants, capable of producing the seeds having the desired oil. The steps of 15 the method are a) selecting a number of Helianthus annuus plants, collecting therefrom the seeds, which have a mutation induced by a mutagenic agent or an introduced transgene such that the oil in the seed has a stearic acid content of at least 12 wt% and preferably 18 wt% 20 based on the oil present in the seed; (b) selecting a number of Helianthus annuus plants, collecting therefrom the seeds, which express an oleic acid content of at least 40 wt% based on the oil present in the seed and a high level of thioesterase activity (c) crossing the 25 plants grown from the seeds of (a) and (b) and, harvesting the F1 seed progeny.

Additional steps include the steps of: (d) 30 planting of the seeds or embryos rescue of the embryos of the F1 progeny obtained to form F2 segregating seeds; (e) selecting from the F2 seeds which developed plants, those plants which produce seeds having an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of the oil, optionally selfing the selected plant to form true 35 breeding inbreds.

The present invention also includes the process for producing F1 hybrid seed. The steps of the method are a) planting seed of two inbreds having high oleic acid

content of at least 40 wt% and high levels of thioesterase activity, one of which may be male sterile, b) crossing the two inbreds, and c) harvesting the F1 seed capable of producing F2 seed with a 40 wt% oleic acid content and a 12 wt% stearic acid content.

#### Description of the invention

The present invention encompasses a vegetable oil with a new and unique fatty acid composition produced in easy to grow crops. The preferred crop is sunflower. However, mutation and/or transformation to produce the derived vegetable oil could likewise modify other crops. These crops would include at least Brassicas, peanuts, palms and other oil producing plants. The crop should be readily transformable and/or susceptible to mutagenically induced oil changes. Rape seed meets all these requirements as does sunflower, these crops are presently some of the most useful crops for production of this new and unique fatty acid composition in the oil of their seeds.

The industrial use for oil which are capable of being extracted from the seeds is determined by its fatty acid composition. Table 2 shows the fatty acid composition and other special properties of some fats and oils. The oils obtained from seeds cultivated in temperate climate (sunflower, soybean, rape seed, etc.) have mainly unsaturated fatty acids, like linoleic and oleic acids, so they are liquid and primarily used for cooking, salad dressing, etc. Saturated fatty acids are often solid at room temperature and referred to as fats. Saturated fatty acid may be obtained from animals (butter, lard, etc.), some tropical trees (cacao, palm) or one chemically modified (hydrogenation and transesterification) liquid vegetable oils. These modified liquid vegetable oils have mainly saturated (palmitic or stearic acids) or chemically modified fatty acids (trans fatty acids) with high melting points.

Table 2 shows the total fatty acid, and other special properties, like trans fatty acid and saturated fatty acid at position 2 of the TAG molecule, of several oils and fats. In the column labelled others are 5 palmitoleic acid in the case of lard and olive oil and also fatty acids shorter than 12 carbons in the butter. Minor amounts (<2%) of other fatty acids are also present in some oils.

Table 2

Oil or fat	Fatty acid composition (%)						Properties	
	Others	Myristic	Palmitic	Stearic	Oleic	Linoleic	Trans	Saturated
Lard	3	2	25	12	45	10	1	79
Butter	14	10	26	12	28	3	3	84
Margarine			10	7	46	34	23	
Palm Oil		1	45	5	39	9		18
Olive Oil	1		14	3	71	10		2
Cocoa Butter			26	35	35	3		4
Normal Sunflower			7	5	30	57		1
High oleic sunflower			5	4	88	2		1

Saturated fatty acids are used by the food industry to make margarine, shortening, bakery, confectionery, snacks, etc. The industry uses these for their physical properties.

5 Unfortunately, saturated fatty acids also have negative nutritional properties. Many fats raise the levels of bad serum cholesterol (low-density lipoprotein, LDL) in blood. The principal component of fats and oils is triacylglycerol (TAG), normally making up more than

95% of the fat or oil. Three fatty acids are bound to a molecule of glycerol to make the TAG. These fatty acids are either saturated or unsaturated. It is believed that negative nutritional properties are i.a. due to having 5 most of the saturated fatty acids in the position 2 of the TAG molecule (figure 1). Most vegetable fats and oils which do not raise LDL have little saturated fatty acids in this position. The fatty acids in positions 1 and 3 are liberated as free fatty acids by lipase in digestion, 10 if these fatty acids are saturated they form insoluble salts with calcium and magnesium, which are mainly excreted. But fatty acids in position 2 form a molecule of monoacylglycerol, which is easily absorbed into the body, raising LDL.

15 Vegetable oils are sometimes modified to form hard fats. These vegetable oils are hydrogenated and/or transesterified to increase the percentage of saturated fatty acids. However, the resulting oils are not necessarily healthier fats. The hydrogenation process 20 produces "trans fatty" acids that may also be less healthy (while the transesterification process randomly exchanges the fatty acids between the three positions). Thus, neither of the two chemical modifications works well to truly form healthy fats.

25 Not all saturated fats are unhealthy, it is believed that cocoa butter, that has around 60% of saturated fatty acids, the rest being mainly oleic acid, does not raise serum cholesterol. This is due to two main reasons, one is that only 4% of the saturated fatty acids 30 are in position 2 and the other is the principal saturated fatty acid is stearic acid. Stearic is considered healthier than palmitic acid, which is the main saturated fatty acid of commodity fats.

35 There remains a need of the industry for a vegetable oil with high stearic acid (as the saturated fatty acid) and elevated oleic acid (as unsaturated fatty acid) content that will resolve the actual fat problems, preferably with the stearic acid in the 2 position of

TAG. To that end the present invention can be selected to have a maximum of 10 wt% of saturated fatty acids in the 2 position. In the more preferred embodiments of the invention there is up to 5 wt% of saturated fatty acids 5 in the 2 position. Thus the present invention produces an oil/fat that is addressing the industry's need for healthier fats.

The main characteristics of modified plants, its seeds and the oil capable of being extracted from 10 these seeds according to the invention are the production of a vegetable oil with increased oleic acid (for sunflowers beyond normal levels as given in Table 1) and with a stearic acid content of 12 wt% and above. Even 15 more specifically the percentage stearic acid content is between 15 wt% and 40 wt% and even more specifically the invention can be selected to have a stearic acid content of between 20 wt% and 35 wt%, an oleic acid content of preferably above 40 wt%. More specifically an oleic acid content of the oil of the present invention is between 50 20 wt% and 80 wt%. Yet, more specifically the present invention has the high stearic acid and the high oleic acid content of between 55 wt% and 75 wt% relative to the total fatty acid content.

Additionally, the invention with high oleic and 25 high stearic acid preferably also contains high levels of thioesterase activity.

The linoleic acid content of the present invention is usually selected to be less than 25 wt% and more preferably less than 20 wt% related to the total 30 amount of fatty acids in the oil. The seeds according to the invention can be selected to have at least 20 wt% total level of saturated fatty acids relative to the total fatty acid content for the oil.

Originally it was believed that crossing the 35 prior art high oleic lines with the prior art high stearic lines would produce an inbred line having high oleic and high stearic properties. However, this proved to be not possible. The prior art high stearic acid

content lines, which were selected after mutagenesis, like CAS-3, evidenced low oleic content. Likewise, most high oleic varieties evidenced low stearic acid content.

It has been found according to the invention  
5 that the selection process for very high oleic material, particularly in sunflower, selects against stearic acid generally. Thus, the present invention is based on the finding that to form a plant such as a sunflower plant, which is capable of producing the desired oil, the  
10 starting material should contain a third characteristic beyond high oleic and high stearic, it should evidence elevated thioesterase activity over the substrate stearoyl-ACP as indicated in Figure 2.

To assist in the understanding of the role of  
15 thioesterase activity within the present invention, a general review of the fatty acid biosynthesis in sunflower is given hereinbelow. The seed fatty acid biosynthesis occurs inside the plastid (Figure 3). The fatty acids within the plastid remain covalently bound to  
20 the acyl carrier protein (ACP) during successive cycles of extension (synthesis of palmitic and stearic acids) and desaturation (conversion of stearic acid to oleic acid). The major products of this biosynthetic process are palmitoyl-ACP, stearoyl-ACP and oleoyl-ACP. A series  
25 of cycling reactions catalysed by the enzymatic complex FAS I produce the palmitoyl-ACP that have 16 carbons. A second enzymatic complex called FAS II elongates the palmitoyl-ACP to stearoyl-ACP (18 carbons), that is further modified by the stearate desaturase to produce  
30 oleoyl-ACP. These are the three main fatty acids synthesised by the plastid. They are separated from the ACP by the action of the thioesterase (Acyl-ACP thioesterase catalyse the hydrolysis of acyl thioesters) and then exported out of the plastid into the cytoplasm  
35 where the synthesis of triacylglycerides takes place. Later the oleic acid can be desaturated to linoleic and linolenic acids. The synthesis of TAG (storage oil) is produced in the cytoplasm using the pool of fatty acids

in the cytoplasm, and this pool is made from the fatty acids exported from the plastid and the linoleic acid made by desaturation. So, the fatty acid composition of TAG is determined by the plastid exported fatty acids 5 plus the linoleic acid produced in the cytoplasm.

A plant capable of producing the oil of the present invention may be obtained by two basic methods. The first method is mutagenesis and breeding the second method is transformation and breeding which is discussed 10 later. Regardless of the method, the trait high levels of thioesterase activity should be identified and bred for in the scope of the present invention.

To form the oil of the present invention a plant capable of producing seed having oil containing the 15 high oleic and high stearic acid content must be formed. To achieve this objective a high oleic, high thioesterase (HOHT) line can be crossed with a high stearic (HS) line which results after selfing and selection in an inbred that is high oleic and high stearic. This requires two 20 basic elements: a high stearic line and a HOHT line. The simplest way to form the present invention is by making sexual crosses of a high stearic line such as CAS-3, with a HOHT sunflower line and selecting for HSHO (a line which produces a seed characterised by high stearic acid 25 and high to medium high oleic acid in the oil). The first basic element CAS-3 is material deposited in the ATCC, the second element is the HOHT.

The first basic element can also be formed by mutation of existing germplasm and screening for the high 30 stearic trait. This can be done according to the teaching of application PCT/EP95/00369 (WO 95/20313). Various mutation methods are detailed in the examples. But briefly one method to form a high stearic acid line treats the parent seeds for 2 hours at room temperature 35 in a solution of 70 mM ethyl methane sulfonate in water, or 2 hours at room temperature with a solution of 2 mM sodium azide in water to form a desired oil trait mutation. The resultant material is selected for the

stearic acid trait. The lines are selfed (if necessary) to form a true breeding line.

The HOHT line can be screened and selected from the existing prior art high oleic sunflower lines. The 5 screening would be based on maintaining the high oleic trait and locating the trait for high thioesterase activity and selecting for both traits. Alternatively the second element, HOHT, can be formed by crossing a fixed high oleic line which is HOLT with a line evidencing high 10 levels of thioesterase activity with or without high stearic properties and through selfing and selection throughout several generations, a new HOHT can be formed or alternatively, a HOHT HS all in one line. A less 15 efficient alternative to forming the HOHT is by mutation, selfing, screening and selection throughout several generations. In this alternative using normal germplasm and mutating it and selecting for either the high oleic trait or the HOHT trait can form the HOHT line. However, since there is a number of high oleic sunflower lines 20 available it should not be necessary to reform a HOHT line from normal germplasm. Screening high oleic lines for high levels of thioesterase activity should be sufficient to locate a HOHT line.

The reason a HOHT line is necessary and the 25 high oleic trait does not appear sufficient is that it has been found that the selection process for very high oleic material selects against stearic generally and also against elevated thioesterase activity over the stearoyl-ACP. Although some high, or more accurately medium-high, 30 oleic lines carry elevated thioesterase activity over the stearoyl-ACP, in general, as the oleic level increases the thioesterase activity decreases. Measuring the thioesterase activity of oleic lines with 85% and above and oleic levels with medium-high levels of 60% - 84% 35 oleic show a number of oleic lines in the medium-high level carry good thioesterase activity over stearoyl-ACP.

Turning to the thioesterase activity over stearoyl-ACP, this activity is used to select the

material for use in the development of new inbred line carrying high oleic and stearic traits according to the present invention. During sunflower seed formation, an active period of fatty acid biosynthesis takes place 5 between 12 and 28 DAF. The maximum *in vitro* acyl-ACP (acyl-carrier-protein) thioesterase activities are found at 15 DAF, preceding the largest accumulation of lipid in the seed (18 DAF). Changes in acyl-ACP thioesterase activity are not only quantitative, but also qualitative, 10 since, although the preferred substrate is always oleoyl-ACP, the affinity for palmitoyl-ACP decreases, whereas that for stearoyl-ACP increases with seed maturation. At 15 DAF the level of thioesterase activity can be measured as shown in the examples to determine which lines should 15 be progressed.

In Table 3 an example is shown of two high oleic lines, the usual high oleic line which has low thioesterase over stearoyl-ACP (HOLT) and the high oleic line with high thioesterase over stearoyl-ACP (HOHT) 20 which has more than twice the thioesterase Vmax over stearoyl-ACP than the usual high oleic line.

Table 3

Sunflower line	Stearic acid (%)	V max
HOHT	17.8	2.03
HOLT	8.0	0.82

Table 3 shows stearic acid content and thioesterase Vmax over the stearoyl-ACP at 15 days after 30 flowering seeds from two high oleic sunflower lines.

This HOHT line was deposited on Sept. 7, 1999 and assigned PTA-628 with the ATCC at 10801 University Boulevard, Manassas, Va 20110-2209. As Table 3 indicates, this line has more stearic acid at 15 days 35 after flowering than the HOLT. This stearic acid level at 15 DAF can also be employed as a rough screening protocol for selecting HOHT lines. The relative affinity of the

enzymes over the stearoyl-ACP standardised with respect to the one over oleoyl-ACP are also shown in Figure 2.

Additionally, the oil obtained from the mature seed of the HOHT also evidences increased stearic acid

5 levels relative to the HOLT line (Table 4).

Table 4

	Fatty acid composition (%)						
	Sunflower line	Palmiti c	Stear ic	Ole ic	Linole ic	Araqui c	Beheni c
10	HOHT	4.3	9.7	78.5	3.9	1.0	2.6
	HOLT	3.8	4.9	84.3	4.8	0.5	1.7

Table 4 shows fatty acid composition (%) of

15 seeds from two high oleic sunflower lines.

Commercially and publicly available high oleic material, the deposited material or newly formed mutated high oleic lines produced according to the examples or by other methods of mutation or transformation can be

20 screened according to the procedure outlined in example

4. The selected lines can have an increased thioesterase Vmax on the substrate stearoyl-ACP at 15 DAF having activity levels as indicated in the definition of high levels of thioesterase. The result of finding a HOHT line 25 is that the fatty acid content will most often evidence an increase in the stearic acid level (when compared to a HOLT) while maintaining a high oleic content. It should be noted that the oleic content of these (HOHT) lines is more likely to be in the range of 60-55%, than above 55%.

30 Previous to the present invention, selecting for high oleic lines selected against this thioesterase activity. Thus, when a HOLT (high oleic line with low thioesterase activity) was crossed with or transformed with a high stearic producing trait the stearic level 35 dropped to unacceptably low levels. However, the HOHT line when crossed with the stearic acid line carries the

stearic acid level of the CAS-3 in the F2 seed. In contrast the HOLT line decreased the stearic acid level of the CAS-3 in the F2 seed. This is shown in Table 5 where lines of both HOLT and HOHT have been crossed with the high stearic CAS-3 line. Also shown in Table 5 is the fatty acid composition of these lines.

Table 5

10		Fatty acid composition (%)					
		Palmitic	Stearic	Oleic	Linoleic	Arauic	Behenic
	HOHT x CAS-3	5.2	24.6	59.2	6.8	1.8	2.4
	HOLT x CAS-3	4.3	17.4	72.1	4.0	1.3	2.8

15 Table 5 shows the fatty acid composition of selected high oleic and stearic F2 lines, with high and low thioesterase activity over stearoyl-ACP, after crossing by CAS-3.

20 In Figures 4 and 5, the F2 segregation for both high stearic acid content and high oleic acid content of the above lines are shown. The seeds formed from the HOHT x CAS-3 cross which have the higher stearic and oleic acids are within a circle. The line with high thioesterase activity over stearoyl-ACP has these kinds of seeds and the line without this high thioesterase activity have no seeds of this type.

25 The selected F2 lines are selfed for 5 to 6 generations in isolated conditions to avoid contamination. The resultant generations are selected, based on high oleic and stearic acid content.

30 Thioesterase activity can be analysed to assist in the selection process. Likewise, marker assisted breeding can be employed to track any or all of the three traits to make the selection process quicker. Various markers such as SSR microsatellite, ASO, RFLP and likewise can be 35 employed. The use of markers is not necessary, as standard tests are known for determining oleic, stearic, and thioesterase activity. However, once identified

markers make trait tracking easier and earlier in the plant's life.

The true breeding plants produce an oil having a similar fatty acid composition to the F2 seeds selected 5 with a low content of saturated fatty acid in the 2 position of the TAG molecule (Table 6).

Table 6

	Fatty acid composition (mol%)					
	Palmitic	Stearic	Oleic	Linoleic	Araquic	Behenic
Total oil	5.5	24.9	57.8	8.2	1.7	1.8
TAG	5.6	26.1	57.6	7.4	1.6	1.7
sn-2 position	1.7	1.9	87.4	9.0	n.d.	n.d.
sn-1 and 3 position	7.2	33.1	46.8	7.3	2.7	2.9

15 Table 6. Fatty acid composition of oil, TAG and Sn positions of true breeding HSHO plants selected. n.d.= not detected.

20 As indicated earlier the two basic elements employed to form the present invention can be developed by mutation and breeding or by transformation and breeding. In accordance with the present invention the sunflower plant can evidence the stearic or oleic acid traits by introduction, either presently or in the ancestry of the line, mutation of the genes. A number of mutation methods including irradiation, chemical mutation, and other forms of causing gene deletions or changes can work. Particularly, easy to use are mutagenic agents that result in small changes in the genome such as 25 point mutations.

30 Alternatively, as mentioned previously there is a second method to produce the stearic or oleic lines in accordance with the present invention, the sunflower plant can evidence the stearic or oleic acid traits by transforming a gene into the plant to produce one or more of the desired traits.

The use of gene sequences to inhibit or promote gene expression is quite well understood. A complete gene

sequence, under the control of a promoter that operates effectively in the plant, will generally overexpress the gene product, leading to an amplification of the effect of the protein so produced. Sometimes the gene product is 5 reduced: this phenomenon is termed "co-suppression".

Reduction of the gene product is also generally obtained by using a dominant negative mutation, or by reversing the orientation of the gene sequence with respect to the promoter so that it produces "antisense" messenger RNA.

10 As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used. The isolation of suitable sequences encoding stearoyl-ACP desaturase or thioesterase from Brassica and 15 Arabidopsis are described in U.S. patent 5,443,974: similar methods may be used to isolate sequences encoding thioesterase or stearoyl-ACP desaturase from other plants. These may have greater or lesser degrees of homology with sequences encoding thioesterase or 20 stearoyl-ACP desaturase from Arabidopsis. Sequences coding for the whole, or substantially the whole, of the protein may thus be obtained. Suitable lengths of this DNA sequences may be cut out for use by means of restriction enzymes. When using genomic DNA as the source 25 of a partial base sequence for transcription it is possible to use either intron or exon regions or a combination of both.

To obtain constructs suitable for modifying expression of the desaturase or thioesterase in plant 30 cells, the cDNA sequence as found in the protein cDNA or the gene sequence as found in the chromosome of the plant may be used. Recombinant DNA constructs may be made using standard techniques. For example, the DNA sequence for transcription may be obtained by treating a vector 35 containing said sequence with restriction enzymes to cut out the appropriate segment. The DNA sequence for transcription may also be generated by annealing and ligating synthetic oligonucleotides or by using synthetic

oligonucleotides in a polymerase chain reaction (PCR) to give suitable restriction sites at each end. The DNA sequence is then cloned into a vector containing upstream promoter and downstream terminator sequences. If 5 antisense DNA is required, the cloning is carried out so that the cut DNA sequence is inverted with respect to its orientation in the strand from which it was cut.

In a construct expressing sense RNA, the template and coding strands retain the assignments and 10 orientations of the original plant gene. Constructs expressing sense RNA encode RNA with a base sequence, which is homologous to part, or all of the sequence of the mRNA. In constructs, which express the functional protein, the whole of the coding region of the gene is 15 linked to transcriptional control sequences capable of expression in plants.

For example, constructs according to the present invention may be made as follows. A suitable vector containing the desired base sequence for 20 transcription is treated with restriction enzymes to cut the sequence out. The DNA strand so obtained is cloned (if desired, in reverse orientation) into a second vector containing the desired promoter sequence and the desired terminator sequence. Suitable promoters include the 25 Actin2 promoter or the pea plastocyanin promoter sequence. Suitable terminator sequences include that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end).

The transcriptional initiation region (or 30 promoter) operative in plants may be a constitutive promoter or an inducible or developmentally regulated promoter, as circumstances require. For example, it may be desirable to modify protein activity at certain stages of the plant's development. Use of a constitutive 35 promoter will tend to affect protein levels and functions in all parts of the plant, while use of a tissue-specific promoter allows more selective control of gene expression and affected functions. Thus the antisense or sense RNA

is only produced in the organ in which its action is required.

The DNA constructs of the invention may be inserted into plants to regulate the expression of the 5 gene encoding for a desaturase or thioesterase or other enzymes in the fatty acid pathway resulting in modification of plant characteristics for production of stearic acid or oleic acid or thioesterase activity in the fatty acid synthesis pathway. Depending on the nature 10 of the construct, the production of the gene product may be increased, or reduced, either throughout or at particular stages in the life of the plant.

A DNA construct of the invention is transformed into a target plant cell. The target plant cell may be 15 part of a whole plant or may be an isolated cell or part of a tissue, which may be regenerated into a whole plant. The target plant cell is preferably sunflower but may be selected from any monocotyledonous or dicotyledonous plant species. The preferred target plant is an oil 20 producing species of monocot or dicot. The more preferred target plant is a Brassica or Helianthus annuus L. Examples of plants which could be modified to produce the desired oil according to the present invention include 25 the oil from oilseed rape (canola), palm, sunflower, soya. Other possible seed transformants are cereals, for example rice and maize, wheat, barley, oats and rye. Plants may be derived from the transformed plant cell by regeneration of transformants and by production of successive generations of the transformants' progeny.

30 Any suitable method of plant transformation may be used. For example, many dicotyledonous plants such as sunflower, oilseed rape, tomato and melon may be transformed by Agrobacterium Ti plasmid technology, such as described by Bevan (1984, Nucleic Acid Research, 35 12:8711 -8721) or Fillatti et al (Biotechnology, July 1987, 5:726-730). Such transformed plants may be reproduced sexually, or by cell or tissue culture. Other

methods use the gene gun, microinjection, PEG and electroporation.

Thus crossing mutants or transgenics or a combination to form seeds containing at least 40% oleic 5 oil and at least 12% stearic oil according to the invention can produce tailor-made seeds and tailor-made oil. The result HSHO lines can be used in further conventional plant improvement practices by crossing with other known or as yet unknown mutant, transgenic or wild 10 type plants.

Alternatively, forming a high stearic line which has a transgene (for example as shown in 5,443,974) that inhibits stearoyl-ACP desaturase activity can make one of the basic elements of the present invention. The 15 gene can be extracted from sunflower material by using the known *Arabidopsis* or the *Brassica* stearoyl-ACP desaturase-encoding gene as taught in U.S. 5,443,974 the sequence of which is in the patent and is incorporated by reference. The known method of transforming sunflower is 20 taught in International application number PCT/US97/01419. This application, unfortunately, does not teach a gene; it does teach how to transform the gene once isolated with a plasmid into the plant genome. The teaching of both of these patents together places the 25 transformation of a desaturase gene into a dicot within the ordinary skilled person's ability. Any of these genes or other similar genes having substantial homology can be placed in either the high oleic line or in a HOHT line that is crossed thereto.

30 The following differences to the standard procedure used to form a transgenic sunflower are herein noted. The preferred method of transformation is the *Agrobacterium* method of transformation. However, due to gene silencing concerns when sunflower is transformed, 35 the promoter should be either an *Arabidopsis* actin gene promoter "Actin2", available from the University of Georgia, Life science Building, Athens Georgia 30602-7223 or possibly a pea plastocyanin promoter which is

published in the LAST & Gray 1989 (Plant Mol. Biol. 12, 655-666) and in Plant Journal (1993) 3(3):437-49. by K. Pwee and J. Gray supplied by the University of Cambridge Dept. of Plant Science Downing Street Cambridge CB23EA or 5 the like. These promoters are more useful in sunflower transformation than 35S or 19s CaMV promoters, though the pea plastocyanin promoter may still show some difficulties.

Thus a high oleic line with elevated 10 thioesterase activity can be transformed with a gene that produces high stearic acid. Although the thioesterase trait is most often discussed in conjunction with the high oleic line it could also be in the high stearic line that is crossed to the high oleic line to form the HSHO 15 line of the present invention. Alternatively, two lines can be transformed, one to have oleic and one to have higher stearic and they can be crossed to form an inbred which is selected for elevated thioesterase activity and both other traits. Or one line can be transformed with a 20 gene overexpressing oleic and/or thioesterase and/or stearic and selected based on fatty acid levels.

Alternatively, a line can be mutated to have both oleic and high stearic with the elevated 25 thioesterase activity in a two step mutation process. Two separate lines can be mutated to carry the oleic and thioesterase trait and the stearic trait and crossed and selfed to form a plant which produces the oil. This would most likely require two inbreds both carrying oleic and stearic oil traits to assure the hybrid formed by 30 crossing these two inbreds had these traits. Once a trait is fixed, through standard breeding or through a marker assisted breeding this trait can readily be moved into different inbreds. By using at least one trait that is transgenic the breeding becomes simpler as transgenes are 35 usually dominant in their inheritance. Thus only one line in a hybrid cross would have to carry the transgene, however, both lines would preferably carry the mutated

traits as these are most often recessive or additive traits not dominant traits.

The seed generated when producing the sunflower capable of producing the oil of the present invention is analysed with a methodology described in Garcés, R. and Mancha, M. "One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues". Analytical Biochemistry, 211:139-143, 1993. This allows for the detection of seeds with modifications in the composition of any fatty acid. Selected seeds showing a desirably high stearic acid content and/or oleic acid and Vmax of thioesterase activity are selected for further breeding purposes. The seeds are cultivated to the fifth generation to form inbreds that have this inheritable and stable genetic trait.

Sunflower oil having a stearic acid content of at least 12% by weight and a oleic acid content of at least 40% by weight, related to the total amount of fatty acids in the oil, is obtainable from sunflower seeds of the invention in any manner known to the person skilled in the art. The sunflower seeds can be converted to crushed seeds or meal before, during or after the oil extraction process. Such extraction methods are well known and for example one method is described in "Bailey's industrial oil and fat products", Vol. 2, Chapter 3; 4th Edition, John Wiley and Sons, New York (1982). The extraction process preferably used does not involve substantial modification of the oil. The extraction process may be selected to avoid substantial chemical, physical or enzymatic rearrangement with no substantial hardening.

In a further embodiment of the method of the invention, the mutation and selection steps may be followed by conventional plant improvement techniques thus leading to seeds having e.g. an even higher stearic acid content or higher oleic acid or reduced linoleic or altered palmitic by weight, or to seeds having a desirable content of one or more other fatty acids. In

still another embodiment the seeds of the invention may be subjected to one or more further mutation treatments or additional transformations.

5 The sunflower plants may be used in breeding programmes for the development of sunflower lines or hybrids, which programmes are aimed at the production of open pollinated or hybrid varieties meeting the requirements of farming practice regarding yield, disease resistance and other agronomically important traits in 10 major sunflower growing areas in the world. Seeds resulting from these programmes may be used in the growing of commercial sunflower crops.

15 The invention further relates to sunflower plants produced from seeds according to the invention. Thus, the seeds can be used to produce new parent lines 20 that have high stearic acid content and a high oleic acid content in their oil. These lines should also have an increased thioesterase Vmax as indicated in the definition of high levels of thioesterase on the substrate stearoyl-ACP at 15 DAF. The seeds may be cultured in the normal way on soil or any other substrate. The production of the modified plants does not require very many additional measures as compared to the growing of ordinary sunflower seeds.

25 The invention also relates to the use in the food industry of sunflower oil extractable from the seeds of the invention. The natural vegetable oil that has been extracted from sunflowers seeds of the present invention 30 has a high stearic acid content between 12 and 35% and at least 40 to 80% oleic acid. This oil can be used directly or it can be combined with other oils, in the production of edible fats or fat mixtures, margarine, vegetable-dairy or in the production of confectionery, cosmetic or 35 bakery goods. The present invention is particularly useful in spreads, sauces, ice cream and soups. The oil is also useful for non-edible products such as lipsticks, soap bars, skin and hair oils, lotions and creams.

In this application the oil of the present invention is interchangeably called oil or fat. The structure of the present invention is in some instances a saturated fatty acid profile that yields a high enough 5 level of saturation to increase the melting point of the oil to make it solid at room temperature. In other saturated fatty acid profiles according to the present invention the material is liquid at room temperature. Both melting points are encompassed by the teachings of 10 the present invention.

This oil is particularly useful in use as a hardstock material for the formation of spreads. This is a fat that has other oils blended into it. In the preferred use the present invention is used as a 15 hardstock at a level of at least 5% depending on the use of the spread the present invention can be used at levels of at least 20%. Higher levels of the present invention can also be employed depending on the traits desired in the resultant spread.

20 A number of food products using the oil of the present invention can be used. These food products would include the present oil at levels from 3% to 100% by weight of food product. The advantage of these oils is that they do not have artificial fatty acid isomers, like 25 the 'trans' isomers found in the hydrogenated oils.

The present invention will be further illustrated by means of the following examples which are given for illustration purposes only and are in no way intended to limit the scope of the invention.

30 In the examples reference is made to the following figures:

**Figure 1:** TAG shown hydrolysis of the triacylglycerols by the lipase;

35 **Figure 2:** is elevated thioesterase activity shown as the relative affinity of the thioesterase on stearoyl-ACP and oleoyl-ACP of HOHT and HOLT;

**Figure 3:** plastid showing the fatty acid biosynthesis in oilseeds;

Figure 4: the F2 segregation for HOHT indicating HOHT progeny;

Figure 5: the F2 segregation for HOLT indicating no HOHT progeny.

5

#### Definitions

"SUNFLOWER" shall mean all Helianthus annuus plant and cell parts including pollen, kernel, oil, embryo, stalk, head, roots, cells, meristems, ovule, 10 anthers, microspores, embryos, DNA, RNA, petals, seeds, and the like and protoplasts, callus or suspensions of any of the above.

"15DAF" shall mean 15 days after flowering.

"HOLT" shall mean a sunflower having high to 15 medium-high (40%-90%) oleic acid levels in the oil when compared to normal, wildtype sunflower seed (oleic acid levels of 17%-20%) levels wherein there is "LOW LEVELS OF THIOESTERASE ACTIVITY".

"TOTAL FATTY ACID CONTENT" of the sunflower oil 20 refers to the sum of C16:0, 18:0, 18:1, 18:2, 20:0, 22:0 and the traces of other like fatty acids as determined simultaneously in the seed sample.

"HOHT" shall mean a sunflower having high to 25 medium-high (40%-90%) oleic acid levels in the oil when compared to normal, wildtype sunflower seed (oleic acid levels of 17%-20%) levels wherein there is "HIGH LEVELS OF THIOESTERASE ACTIVITY".

"LOW LEVELS OF THIOESTERASE ACTIVITY" shall 30 mean levels of stearoyl-ACP thioesterase activity at DAF15 which are lower than 10% of the activity over oleoyl-ACP, this permits an exportation out of the plastid of a maximum of less than 12% of stearic acid with respect to the total fatty acids synthesised by the plastid.

"HIGH LEVELS OF THIOESTERASE ACTIVITY" shall 35 mean levels of stearoyl-ACP thioesterase activity at DAF15 which are higher than 10% of the activity over oleoyl-ACP, this permits an exportation out of the

plastid of a minimum of 12% of stearic acid with respect to the total fatty acids synthesised by the plastid.

"HIGH STEARIC LINE" shall mean a sunflower having increased stearic acid levels in the oil (12% and 5 above) and even more preferably 18% and above when compared to normal wild type sunflower seed.

"HOHS" shall mean sunflower comprising above normal levels of oleic acid preferably having levels of above 40% oleic acid in the oil and comprising above 10 normal levels of stearic acid found in wild type sunflower seeds at least 12% stearic acid in the oil and preferably having levels of above 18% stearic acid in the oil found in wild type sunflower seeds.

15 **EXAMPLES**

**GENERAL**

The invention also relates to a method for preparing sunflower seeds having an increased stearic acid and oleic acid content as compared to wild type seeds. This method includes the step of treating parent seeds with a mutagenic agent during a period of time and in a concentration sufficient to induce one or more mutations in the genetic trait involved in stearic acid or oleic acid biosynthesis. This results in an increased 20 production of stearic acid and/or an increased level of oleic acid. These mutagenic agents include agents such as sodium azide or an alkylating agent, like ethyl methane sulfonate, of course any other mutagenic agent having the same or similar effects may also be used. The treated 25 seeds will contain inheritable genetic changes. These mutated seeds are then germinated and progeny plants are developed therefrom. To increase the traits in the lines the progeny can be crossed or selfed. The progeny seeds are collected and analysed. Seeds having the HOHT trait 30 or the stearic trait can then be crossed to each other to form the HSHO line. Optionally there can be additional cycles of germination, culturing, and selfing to fix the 35

homozygosity of the traits in the lines and crossing and collection of seeds.

Sodium azide and ethyl methane sulfonate were used as mutagenic agents in Example 1 and 2,

5 respectively. Several sunflower lines with a stearic acid content between 12 and 45% have been obtained. In all these cases the original sunflower parent line for the production of the high stearic acid lines used was RDF-1-532 (Sunflower Collection of Instituto de Agricultura 10 Sostenible, CSIC, Cordoba, Spain) that has from 4 to 7% stearic acid content in the seed oil. The high oleic material used herein is derived from the Russian researched oleic lines. It is sufficient to screen oleic lines for a HOHT and use this line for either 15 transformation or for crossing to a high stearic line to develop a HSHO line. A method for the preparations of the stearic lines, and of oleic lines if necessary, have been described in the following examples.

20 **EXAMPLE 1**

Seeds were mutagenized with a solution of 70 mM of ethyl methane sulfonate (EMS) in water. The treatment was performed at room temperature during 2 hours while shaking (60 rpm). After mutagenesis the EMS solution was 25 discarded and seeds were washed during 16 hours under tap water.

Treated seeds were germinated in the field and plants were self-pollinated. The seeds collected from these plants were used to select new sunflower lines with 30 modifications in the fatty acid composition. By using the method of Garcés, R. and Mancha, M. (supra) the seed fatty acid composition was determined by gas liquid chromatography, after converting the fatty acids into their corresponding methyl esters.

35 A first plant with 9 to 17% stearic acid content in the oil was selected. The progeny was cultivated for five generations wherein the stearic acid content increased and the new genetic trait became stably

fixed in the genetic material of the seed. This line is called CAS-3. The minimum and the maximum stearic acid content of the line were 19 and 35% respectively. The stearic acid content of oil extracted from seeds from 5 this cell line may thus lie between 19 and 35%.

#### EXAMPLE 2

Sunflower seeds were mutagenized with sodium azide, at a concentration of 2 mM in water. The treatment 10 was performed at room temperature during two hours while shaking (60 rpm). Then the mutagenesis solution was discarded and seeds were washed during 16 hours with tap water.

Seeds were planted in the field and plants were 15 self-pollinated. Seeds from these plants were collected, and the fatty acid composition was determined by gas liquid chromatography, after converting the fatty acids into their corresponding methyl esters using the method described in Example 1.

Seeds from a plant having around 10% stearic acid in the oil were selected and cultivated for five generations. During this procedure the stearic acid content was increased and the new genetic trait fixed. This line is called CAS-4. A selected sample of this line 25 was analysed resulting in a stearic acid content of 16.1%. The minimum and the maximum values were 12 and 19%, respectively.

Table 7

Line	Percentage fatty acids			
	Palmitic	Stearic	Oleic	Linoleic
CAS-3	5.1	26.0	13.8	55.1
CAS-4	5.5	16.1	24.3	54.1

**EXAMPLE 3**

Sunflower plants were grown from the sunflower seeds of the HOHT line, seeds of which are on deposited at ATCC, shown in Table 4. Sunflower plants were also 5 grown from the sunflower seeds of CAS-3. The lines were crossed. The plants were assisted by artificial pollination in order to ensure adequate seed production occurred. The F1 seed was produced on the HOHT line, or vice versa, and harvested. The F2 seeds with more than 10 20% stearate and more than 40% oleate were selected. Although this produces the oil of the present invention the level of production is limited. Therefore fixed inbred lines evidencing seeds with these oil profiles are desirable. These homozygous fixed inbred HSHO lines can 15 then be crossed to form hybrid seed, which will produce F2 seed evidencing the desired oil traits of the present invention. Toward this end the F1 seeds were planted and produced plants were selfed in isolated conditions and F2 seed was produced. The F2 seed was tested for the three 20 traits, high stearic, high oleic and high levels of thioesterase activity. The remaining portion of the seeds evidencing these traits was employed to grow plants to form F3 seed. The selfing and screening and selection process is repeated to develop the fixed homozygous HSHO 25 line, having the following fatty acid profile, C:16 5.4, C:18.0 24.8, C:18.1 58.5, C:18.2 7.2. Once the trait is fixed similar HSHO lines can cross to form hybrid seed having both traits.

According to the invention sunflower plants and 30 seeds from which said oil can be extracted have been obtained by means of a biotechnological process. This high stearic acid content is an inheritable trait and is fairly independent from the growing conditions.

**EXAMPLE 4**Plants growth conditions

Sunflower (Helianthus annuus L.) seeds from high oleic lines with altered seed fatty acid content was

used to test for the thioesterase activities over  
stearoyl-ACP. Plants were cultivated in growth chambers  
at 25/15°C (day/night) temperature, 16 hours photoperiod  
and photon flux density of 300 micromol m-2s-1. Seeds for  
5 analysis were harvested at 15 days after flowering and  
kept at -20°C.

Radioactive reagents and preparation of acyl-ACPs

10  $1^{-14}\text{C}$ -Oleic with specific radioactivity of 2.1  
GBq/mmol and [9,10(n)- $3\text{H}$ ] stearic acid with specific  
radioactivity of 1.9 GBq/mmol were obtained from American  
Radiolabeled Chemicals Inc. (St.Louis, Mo., USA). To  
prepare the fatty acid sodium salt, an appropriate volume  
of fatty acid solution was transferred to a glass tube,  
15 the solvent was removed under a stream of nitrogen, and  
the residue was dissolved in 10% Triton X-100, 0.6 mM  
NAOH. This solution was heated at 55°C for 1 hour to  
ensure homogeneity. Acyl-ACPs were prepared using a  
modification of the enzymatic synthesis procedure of Rock  
20 CO, Garwin JL, Cronan, JE Jr. (1981) Preparative  
enzymatic synthesis of acyl-acyl carrier protein. Methods  
Enzymology 72:397-403. Assays contained 0.1 M Tris-HCl  
(pH 8.0), 0.4 M LiCl, 5 mM ATP, 10 mM MgCl<sub>2</sub>, 2 mM DTT,  
130 microM fatty acid sodium salt, 0.27 mM ACP-SH and 1.8  
25 mM of acyl-ACP synthetase (the last two components were  
purchased from Sigma-Aldrich Quimica S.A. Madrid, Spain)  
in a final volume of 110 microliter. Reactions were  
incubated at 37°C for 3 hours. After this time the pH was  
acidified to 6.0 by adding 1 microliter of 3.6 M HCl and  
30 the mixture was cleaned of free fatty acids using a  
modification of the method described by Mancha M, Stokes  
GB, Stumpf PK (1975) Fat metabolism in higher plants. The  
determination of acyl-acyl carrier protein and acyl  
coenzyme A in a complex lipid mixture 1,2. Anal. Biochem.  
35 68:600-608. Consisting in adding an equal volume of  
isopropanol and washing three times with hexane saturated  
in water/isopropanol (1:1; v/v).

Preparation of crude extracts for enzyme assays and protein determination.

Frozen seeds were peeled and ground in extract buffer containing 20 mM Tris-HCl (pH 8.5), 2 mM DTT and 5 5% (v/v) glycerol (Dörmann P, Kridl JC, Ohlrogge JB (1994) Cloning and expression in *Escherichia coli* of a cDNA coding for the oleoyl-acyl carrier protein thioesterase from coriander (*Coriandrum sativum* L.). *Biochim. Biophys. Acta* 1212:134-136) at 1 g of tissues/ 10 10 ml of buffer. Protein concentrations were measured using a Protein Assay Kit (Bio-Rad) according to the manufacturer's recommendations, with BSA as standard.

#### Enzyme assays

15 Acyl-ACP thioesterase activity was assayed in a final volume of 170 microliter using 130 microliter of crude extract. Control assays had crude extract omitted. Reactions mixtures contained 20 mM Tris-HCl (pH 8.5), 5% glycerol and 2 mM dithiothreitol (DTT) and different 20 concentrations of substrates (stearoyl-ACP and oleoyl-ACP). Incubations were carried out for 20 min at 25°C. Reactions were stopped by the addition of 170 microliter of 1 M acetic acid in isopropanol containing 1 mM of oleic acid. Mixtures were then washed three times with 25 hexane saturated in water/isopropanol (1:1, v/v). Acyl-ACP thioesterase activity was determined by counting the radioactivity of the aqueous phase, which contained the non-hydrolysed substrates. Then, 3 ml of solvent scintillant (purchased from National Diagnostics, Hessle, 30 England) was added and the radioactivity was measured using a scintillation counter (Rackbeta II; LKB, Sweden). Data from acyl-ACP thioesterase assays were fitted to the Michaelis-Menten equation by non-linear least-squares 35 regression analysis using Microcal Origin 4. 1, and correlated to P<0.05, as determined by paired Student's test. Vmax and Km were derived from these curves.

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## 1 CLAIMS

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1. Plants which form seeds which contain an endogenous oil wherein the levels of stearic acid 5 (C:18) and oleic acid (C:18:1) are provided in a combination that is different from the combination found in existing plants.

2. Plants as claimed in claim 1, wherein the oil comprises an oleic acid content of more than 40 10 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of said oil.

3. Plant as claimed in claim 1 or 2, wherein the oleic acid content is from 55 to 75 wt%.

4. Plant as claimed in claims 1-3, 15 wherein the stearic acid content is from 15 to 40 wt%.

5. Plant as claimed in claims 1-4, having a total level of saturated fatty acids of at least 20 wt%.

6. Plant as claimed in claims 1-5, 20 having a linoleic acid content of less than 20 wt%.

7. Plant as claimed in claims 1-6, wherein the a maximum of 10 wt% of the saturated fatty acid groups in the oil are in the 2 position of a triacylglyceride (TAG) molecule.

25 8. Plant as claimed in claim 7, wherein up to 5 wt% of the saturated fatty acid groups are in the 2 position of a TAG molecule.

9. Plant as claimed in claims 1-8, which plant is a sunflower plant.

30 10. Seeds produced by plants as claimed in claims 1-9.

11. Seeds as claimed in claim 10, which seeds are sunflower seeds.

35 12. Oil obtainable from seeds produced by plants as claimed in claims 1-9 or from seeds as claimed in claims 10 and 11.

13. Oil as claimed in claim 12, said oil comprising, an oleic acid content of more than 40 wt% and

a stearic acid content of more than 12 wt% based on the total fatty acid content of said oil.

14. Oil as claimed in claims 12 and 13, wherein the oleic acid content is from 55 to 75 wt%.

5 15. Oil as claimed in claims 12-14, wherein the stearic acid content is from 15 to 40 wt%.

16. Oil as claimed in claims 12-15, having a total level of saturated fatty acids of at least 20 wt%.

10 17. Oil as claimed in claims 12-16, having a linoleic acid content of less than 20 wt%.

18. Oil as claimed in claims 12-17, wherein a maximum of 10 wt% of the saturated fatty acid groups in the oil are in the 2 position.

15 19. Oil as claimed in claim 18, wherein up to 5 wt% of the saturated fatty acid groups are in the 2 position.

20 20. Oil as claimed in claims 12-19 which oil is a sunflower oil.

21. Method for producing a plant which forms seeds that comprise an endogenous oil wherein the levels of stearic acid (C:18) and oleic acid (C:18:1) are provided in a combination that is different from the combination found in existing plants, which method comprising, which method comprises:

a) providing seeds which contain an oil having a stearic acid content of at least 15 wt%;

30 b) providing seeds which contain an oil having an oleic acid content of at least 40 wt% and a high level of thioesterase activity;

c) crossing plants grown from the seeds provided in step (a) and (b);

d) harvesting the F1 seed progeny.

22. Method as claimed in claim 21, 35 further comprising the steps of:

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

5 g) testing the seed for the presence of a stearic acid content of at least 15 wt%, an oleic acid content of at least 40 wt% and a high level of thioesterase activity;

h) planting seeds having the desired levels of stearic acid content, oleic acid content and thioesterase activity to grow plants;

10 i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic acid content, oleic acid content and thioesterase activity are fixed.

15 23. Method as claimed in claims 21 and 22, wherein the seeds which contain an oil having a stearic acid content of at least 15 wt% are provided by:

a) mutagenic treatment of seeds having a stearic acid content of less than 15%;

20 b) producing plants therefrom which are pollinated to produce seeds;

c) testing the seeds for the desired stearic acid content; and

d) optionally repeating steps b) and c).

25 24. Method as claimed in claims 21-23, wherein the seeds are sunflower seeds.

26. Method for producing an oil having an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of said oil by extracting oil from the seeds according to claims 12-20.

27. Method according to claim 25, wherein said extraction process does not involve a substantial modification of the oil.

35 28. Method according to claim 26, wherein no substantial chemical or enzymatic rearrangement takes place and no substantial hardening.

28. Method as claimed in claims 25-27  
wherein the oil is sunflower oil.

29. Food product comprising a sunflower oil, said oil having an oleic acid content of more than 5 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of said oil.

30. Food product as claimed in claim 29, wherein the oil is obtainable from seeds as claimed in claims 12-20.

10 31. Food product as claimed in claims 29 and 30, wherein the level of sunflower oil is from 3 to 100 wt%.

32. Food product as claimed in claims 29-31 selected from the group of spreads, sauces, ice-15 cream, soups, bakery products and confectionery products.

33. Food product according to claim 29-32, being a spread in which the sunflower oil is used as a hardstock at a level of 5 to 20 wt%.

34. Cosmetic product comprising a 20 sunflower oil, said oil having an oleic acid content of more than 40 wt% and a stearic acid content of more than 12 wt% based on the total fatty acid content of said oil, wherein the oil is obtainable from seeds as claimed in claims 12-20.

25 35. Cosmetic product as claimed in claim 34, wherein the level of sunflower oil is from 3 to 100 wt%.

36. Cosmetic product as claimed in claims 34 and 35 selected from the group of creams, 30 lotions, lipsticks, soap bars and skin or hair oils.

37. Products as claimed in claims 29-36, wherein the oil is a sunflower oil.

38. Meal or crushed seeds obtainable from the seeds according to claims 10 and 11.

35 39. Meal or crushed seeds as claimed in claim 38, wherein the seeds are sunflower seeds.

40. An inbred plant having seeds with oil according to claims 12-20 including that said seeds

have a thioesterase activity over stearoyl-ACP of at least 10% of the activity over oleyl-ACP.

41. An inbred plant as claimed in claim 40, including a maximum of 10 wt%, preferably up to 5 wt% of saturated fatty acids in the 2 position of a TAG molecule.

42. A hybrid plant obtainable by crossing a first parent plant resulting from the method as claimed in claims 22 and 23 and a second parent plant 10 having desirable characteristics.

43. A hybrid plant as claimed in claim 42, wherein the first parent is a plant as claimed in claim 40 or 41.

44. Plants as claimed in claims 40-43, 15 which plant is a sunflower plant.



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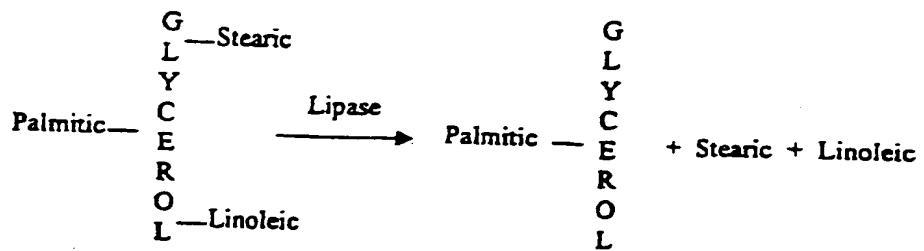


Figure 1.

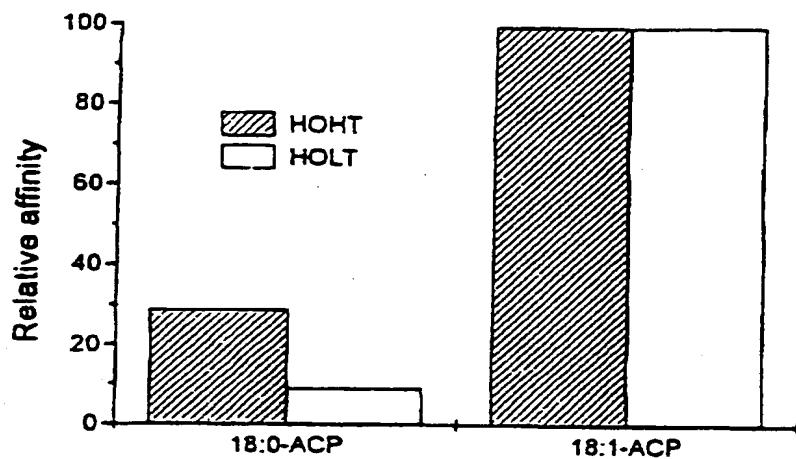


Figure 2..

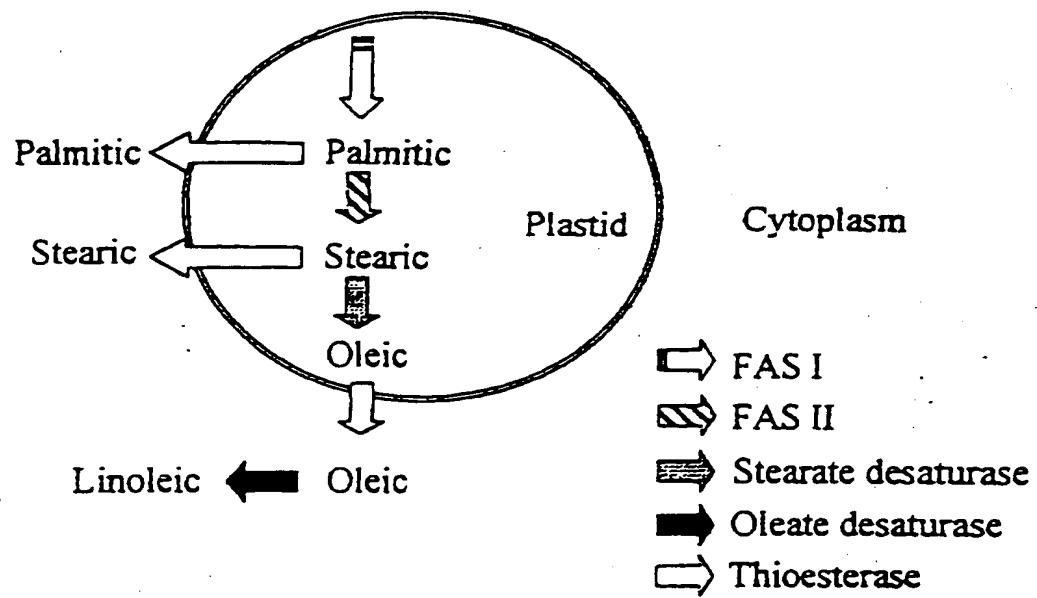


Figure 3.

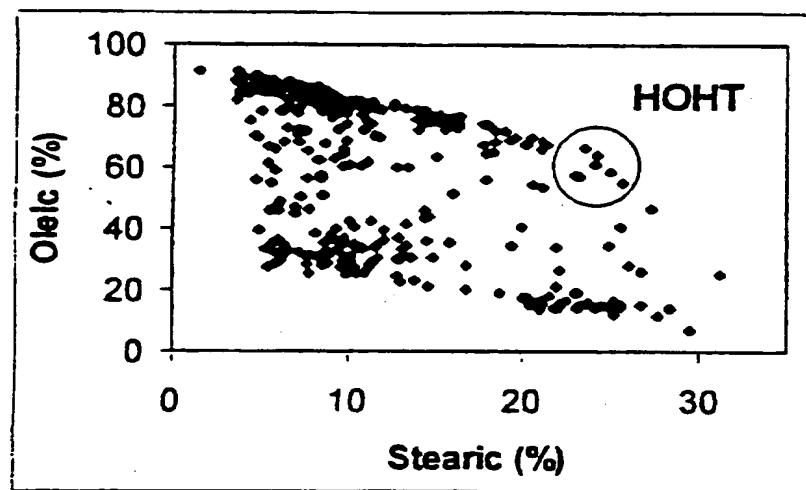


Figure 4.

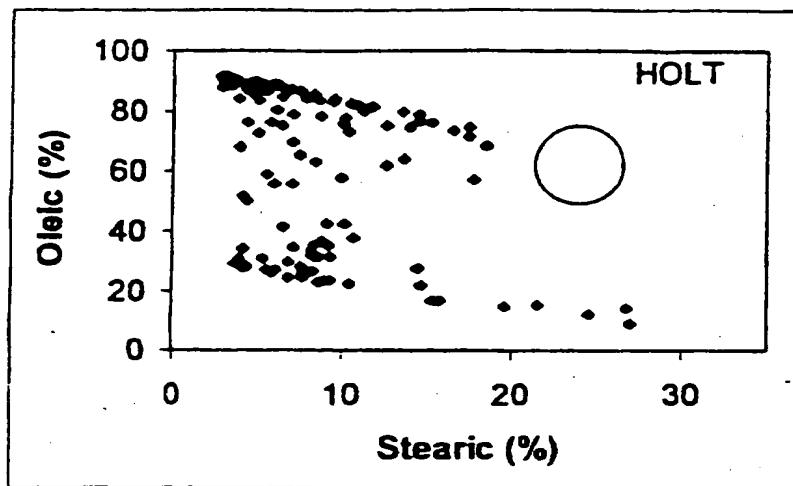


Figure 5.

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